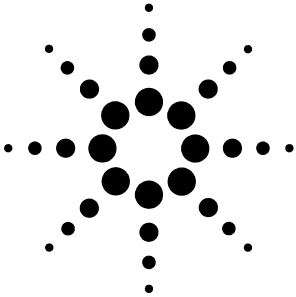
Agilent Plant RNA Isolation Mini Kit



Protocol

Product Number 5188-2780

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May 2005

Store kit and all reagents at room temperature.

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1 Introduction

Product Description

The Agilent Plant RNA Isolation Mini Kit provides a phenol-free, spin column technique for the preparation of concentrated, highly purified, intact total cellular RNA (see **Figure 1**). This kit can be used for isolation of total RNA from many different plant species and tissue types (see **Figure 2**). Recoveries of total cellular RNA are comparable to or greater than those obtained by typical silica or glass fiber methodologies. Typical results are shown in **Table 1**.

An important advantage of this rapid and convenient method is the removal of contaminating genomic DNA (gDNA) from the isolated total cellular RNA. Virtually all gDNA is removed in the initial stage of the protocol by centrifuging the cell or tissue homogenate through a unique mini prefiltration column. Typically, this method results in a 1000- to 10,000-fold lower level of gDNA, relative to typical silica or glass fiber isolation methods for plant material. For the majority of downstream applications, including microarray gene expression analysis, no supplemental DNase treatment step is necessary when using total RNA purified by this method. An additional advantage of the kit, for applications requiring high RNA concentrations, is the relatively small elution volume for the isolation column - as little as 10 µL of water. Total RNA is recovered with high yield, and without the addition of buffers or solvents that may interfere with subsequent enzymatic, chemical, or physical manipulations.

The intact and highly-purified RNA prepared by this method is ideal for cDNA synthesis and labeling, RT-PCR and quantitative RT-PCR, Microarray Gene Expression experiments, Northern Blotting, RNase protection assays and other downstream applications.

Homogenize tissue or cells

1 Prepare tissue or seed homogenate in appropriate volume of Extraction Solution/β-ME mixture.

Spin through prefiltration column

2 Centrifuge up to 600 µL of homogenate through the mini prefiltration column (natural) for 3 minutes at 16,000 × g.

Capture RNA on mini isolation column

3 Add an equal volume of isopropanol to the filtrate and mix until homogeneous. Incubate 5 minutes.

4 Apply to mini isolation column (blue) and centrifuge 30 seconds at 16,000 × g. Discard flow-through and replace RNA-loaded column in same collection tube.

Wash two times and spin dry

5 Add 500 µL of prepared Wash Solution to the mini isolation column and centrifue 30 seconds at 16,000 × g. Discard flow-through and replace isolation column in the same collection tube.

6 Add 500 µL wash solution and centrifuge 2 minutes at 16,000 × g.

Elute pure intact RNA

7 Elute the purified RNA by adding 10–50 µL of nuclease-free water, waiting 1 minute, and centrifuging 1 minute at 16,000 × g.

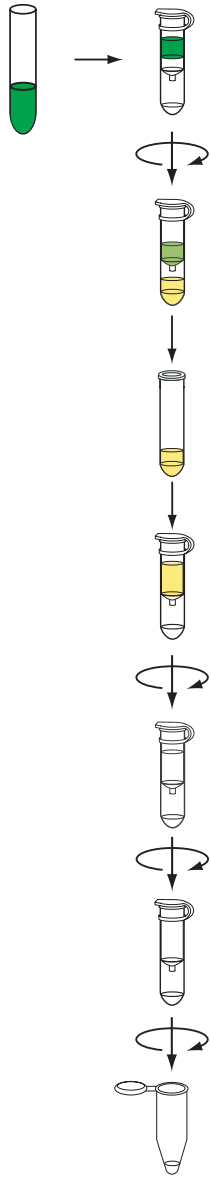

Figure 1 Agilent plant RNA isolation method

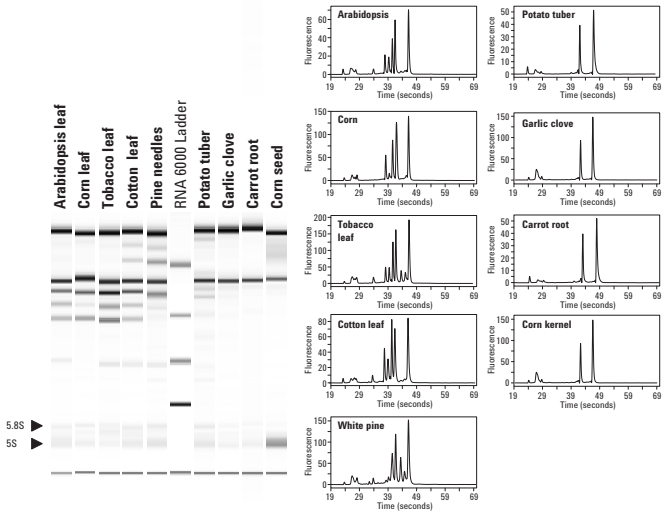

Figure 2 Typical Agilent Technologies Bioanalyzer scan of total RNA isolated from various plant species and tissue types.

Table 1 Typical yields of total cellular RNA

Leaf tissues	A_{260}/A_{280}	A_{260}/A_{230}	Yield* (ng/mg tissue)	gDNA† (pg/µg NA)
Arabidopsis	2.11	2.29	512	0.21
Corn	2.13	2.31	346	1.92
Rice	2.11	2.35	1104	6.26
Wheat	2.14	2.35	437	0.75
Cotton	2.10	1.99	217	11.51
Tobacco	1.88	1.84	280	0.77
Cucumber	2.10	2.39	959	2.56
Tomato	2.12	2.30	613	0.13
White pine (needles)	2.11	2.20	214	3.25
Nonphotosynthetic tissue				
Corn root	2.12	2.14	92	5.35
Arabidopsis root	2.12	2.24	330	ND

* Determined by optical absorbance at 260 nm, in 10 mM Tris/1 mM EDTA, pH 8 (40 µg/mL RNA per unit A_{260}). Recoveries for tissues are typical for a 40-mg sample. Yields may vary significantly depending on developmental stage and environmental conditions.

† Determined by real-time PCR with a primer/probe set specifically for th e18S rRNA gene using Arabidopsis thaliana DNA as a standard.

ND Not determined

Kit Components

The Agilent Plant RNA Isolation Mini Kit includes the components listed in Table 2.

Table 2 Kit contents

Amount	Component
50 mL	Extraction Solution
19.5 mL	Wash Solution
25 mL	Nuclease-free water
50 each	Mini isolation columns (blue) paired with 2-mL collection tubes
50 each	Mini prefiltration columns (natural) paired with 2-mL collection tubes
50 each	1.5-mL RNase-free final collection tubes
1	Agilent Plant RNA Isolation Mini Kit Protocol
1	Agilent Plant RNA Isolation Protocol Quick Reference Card

Additional Materials Required

In order to perform the protocols, you will also need the materials listed in Table 3:

Table 3 Additional materials required

Materials needed
100% Ethanol
Isopropyl alcohol
14.3 M β-Mercaptoethanol (neat) (β-ME)
Microcentrifuge
Nuclease-free water
Tissue homogenizer

Reagent Storage

Store all reagents at room temperature. After addition of β-ME, the Extraction Solution is good for 1 month at 4 °C (see page 11).

Ordering Information

Re-order part number 5188-2780, Agilent Plant RNA Isolation Mini Kit (50).

Related Products

Agilent Mini Prefiltration Columns and Collection Tubes (50)	5188-2736
Agilent Low Input RNA Fuorescent Linear Amplification Kit	5184-3523
Agilent Fluorescent Direct Label Kit	G2557A
Agilent cRNA Cleanup Kit	5188-2770
Agilent Arabidopsis 2 Oligo Microarray Kit	G4136A
Agilent Arabidopsis 3 Oligo Microarray Kit	G4142A
Agilent Rice Microarray Kit	G4138A
60-mer Custom Oligonucleotide Microarray - 8.4K format	G2508A
60-mer Custom Oligonucleotide Microarray - 22K format	G2509A

For more informaiton on these and other Agilent products, please visit our Web site: **http://www.chem.agilent.com**.

2 Preparation

Reagents

Before beginning the following protocol, prepare the reagents listed below.

Wash Solution

To prepare the Wash Solution, add 45.5 mL of ACS grade 100%, ethanol to the provided bottle labeled **Wash Solution**. Mix well. Check the box on the bottle label to indicate that ethanol has been added.

Extraction Solution

Prepare by adding 500 µL of β-mercaptoethanol (β-ME) to the bottle containing Extraction Solution. To prepare smaller amounts, add 10 µL of β-ME per 1 mL of Extraction Solution.

Store the Extraction Solution at 4 °C after the addition of β-ME. This solution is good for 1 month at 4 °C.

WARNING **Extraction Solution contains guanidine hydrocholoride, which may be harmful if inhaled, swallowed, or absorbed through the skin. Guanidine hydrocholoride causes skin, eye, and respiratory irritation. Do not mix with bleach or acids. Wear gloves when using the Extraction Solution and follow your institution's policies for chemical hygiene and disposal.**

RNase-Free Technique

Since RNases are extremely stable enzymes, it is very difficult to inactivate them. A policy of avoidance is central to successful work with RNA. The following general principles will help you avoid RNase contamination of your RNA sample:

- Wear clean laboratory gloves at all times during the RNA isolation procedure and whenever handling lab equipment and any materials involved in the method.
- Clean the lab bench, pipettors, homogenizer, and other equipment with RNase decontamination solutions. Rinse well with nuclease-free water.
- Use only RNase-free filtered pipette tips and RNase-free microcentrifuge tubes.
- If possible, use sterile disposable plasticware. Otherwise, decontaminate glassware by baking it overnight at 180 °C.
- Clean and decontaminate (for example, by treatment with 3% hydrogen peroxide) any electrophoresis gel boxes, then rinse with RNase-free water.

3
RNA Isolation Protocol

This chapter describes the protocol available for the Plant RNA Isolation Mini Kit, **"Agilent Plant RNA Isolation Protocol for Most Plant Tissues" on page 14**.

Agilent Plant RNA Isolation Protocol for Most Plant Tissues

This method is designed for mini scale RNA isolation from plant tissue, and is optimized for 25–100 mg of tissue samples. Some tissue, such as desiccated seeds, require much less material. Typical yields of RNA for a variety of species and tissue types are shown in Table 1.

NOTE **Columns included**
This kit includes two types of color-coded columns: a mini prefiltration column (natural) and a mini isolation column (blue). Be sure to use the correct type as specified by the protocol.

NOTE All steps are to be done at **room temperature**. All centrifugation steps are done in a microcentrifuge at full speed (approximately 16,000 × g).

WARNING **To avoid injury, wear protective eyewear when using liquid nitrogen.**

Be sure that reagents are properly prepared before starting procedure

Extraction Solution

Prepare by adding 1/100 volume of β-ME (10-µL βME/mL Extraction Solution) to the Extraction Solution. This working stock of Extraction Solution may be prepared as needed or the entire bottle may be prepared by adding 500 µL of β-ME. Store the working stock of Extraction Solution at 4 °C after the addition of β-ME.

Wash Solution

Prepare the Wash Solution by adding 45.5 mL of ACS grade 100% ethanol to the provided bottle labeled Wash Solution. Mix well. Be certain to check box on the bottle label after adding alcohol.

Tissue preparation and homogenization

NOTE Plant tissue can be processed using several different methods. The method described below uses a rotor-stator type homogenizer (Omni International TH homogenizer or Brinkmann Polytron homogenizer) and has been optimized for this kit. Other methods, such as grinding in liquid nitrogen, a Dounce homogenizer or microcentrifuge tube mini-pestles, will also work. Complete homogenization is the critical parameter for optimal yield.

Table 4 Recommended volumes of Extraction Solution for tissue homogenization

Tissue type	Volumes Extraction Solution (µL/mg tissue)
Most tissue	5–10
Low water content tissue (high biomass)	10–20
High starch (storage tissue)	10–20
Desiccated seed	20–40

1 **Collect the tissue sample.** RNA degradation can start as soon as the tissue is harvested, thus it is important to work quickly with fresh material or flash freeze in liquid nitrogen. Flash freezing can be especially useful if several samples are to be harvested. Flash-frozen tissue can also be stored at –70 °C for future use.

2 **Prepare tissue for homogenization.** Fresh material should be small enough to enter the blades of the rotor-stator probe. Fresh leaf material should be cut into 0.5-cm squares with a razor blade. This is especially important for fibrous tissue such as corn leaf. Frozen tissue can be crushed directly in the tube using a metal spatula or glass rod prechilled in liquid nitrogen. It is important to keep the tissue frozen until buffer is added and homogenizing immediately.

3 **Weigh the sample** (25 to 100 mg) and place **in appropriate sized tube**. A 15-mL round-bottom polypropylene tube works well for volumes between 1 and 8 mL.

4 **Add extraction buffer**. Use 10 µL of Extraction Solution (containing 1% β-ME) per milligram of tissue to be homogenized and **proceed to homogenization (step 5) immediately**.

If higher RNA yields are desired, five volumes of Extraction Solution may be used. However, for some tissue, especially those low

in water content or high in starch content, may overload the prefiltration column (see Table 4).

5 **Immediately and vigorously homogenize tissue**. A conventional rotor-stator homogenizer at 15,000 rpm (this is 50% of full speed for an Omni International TH homogenizer) for 30–60 seconds works well for most tissue types. Other methods, such as grinding in liquid nitrogen, Dounce homogenizer, or microfuge minipestle, will also work. To reduce foaming, move the probe from side to side rather than up and down. Larger volumes (more than 10 mL) or fibrous tissues may require slightly longer homogenization times. Incomplete homgenization can lead to reduced yields and may clog the prefiltration column.

6 **Apply 600 µL of homogenate** (equivalent to 60 mg of tissue) **to a mini prefiltration column** (**natural**). Wide-bore pipet tips are recommended for transfer since the homogenate will often contain particles and cell debris that plug standard tips. For volumes of homogenate exceeding 600 µL, additional prefiltration columns must be used. Mini prefiltration columns cannot be reused.

7 **Centrifuge** for 3 minutes at full speed (for a typical microcentrifuge, approximately 16,000 × g). This step ensures complete homogenization of the tissue, removes gDNA and other cellular contaminants. The flow-through should appear clear or slightly yellow. A green flow-through from photosynthetic tissue is a good indicator of incomplete homogenization and filter clogging. In this case the RNA yield and quality is still high but the gDNA contamination may be higher than normal.

8 **Add an equal volume of isopropyl alcohol to the filtrate**, mix by pipetting up and down 2–3 times and transfer to a mini isolation column (blue). Use a volume equal to the amount of homogenate initially added to the prefiltration column in step 6. The prefiltration column removes cell debris eliminating any pellet in the collection tube. This allows for mixing and transfers directly from collection tube to the isolation column without transfer to a clean tube. This also eliminates potential contamination with pellet material. **Incubate this mixture for 5 minutes.**

9 **Centrifuge the homogenate/isopropyl alcohol mixture** for 30 seconds at 16,000 × g. Discard the flow-through, and replace the RNA-loaded column in the collection tube. If the isopropyl alcohol/homogenate mixture volume exceeds 600 µL, add aliquots successively onto the mini isolation column, then centrifuge and discard the flow-through as described above.

10 **Add 500 µL of prepared Wash Solution to the mini isolation column**. Centrifuge for 30 seconds at 16,000 × g. Discard the flow-through, then replace the mini isolation column in the same collection tube.

11 **Add 500 µL of prepared Wash Solution to the mini isolation column.** Centrifuge for 2 minutes at 16,000 × g. The 2-minute spin ensures that the mini isolation column is completely dry and no residual ethanol is carried over during the elution. Discard the flow-through and place the mini isolation column in a clean 1.5 mL RNase-free collection tube.

12 **Add** 10–50 µL of nuclease-free water to the top center of membrane (without touching membrane). **Incubate 1 minute**, then **centrifuge** for **1 minute** at 16,000 × g.

NOTE If more concentrated RNA samples are desired for downstream application, the elution volume may be as low as 10 µL. However, if the final RNA concentration exceeds 3 µg/µL, quantitative recovery of the RNA may be compromised. The expected yield must be taken into consideration when choosing an elution volume. If the expected yield is not known, use 10 µL nuclease-free water for elution and determine the concentration by $A_{260\ nm}$. If the concentration is greater than 3 µg/µL, residual RNA may be remaining on the membrane. Apply an additional 10 µL to the isolation column, spin 1 minute at 16,000 × g and determine concentration of the second elution. Pool with the initial elution if significant RNA is present in the second elution.

4 Quality Analysis and Quantification of Total RNA Samples

This section provides guidance for determining the quality and yield of total RNA collected with this kit using three methods: UV absorption, the Agilent 2100 Bioanalyzer, and gel electrophoresis.

UV Absorbance

Spectrophotometry may be used to determine the nucleic acid concentration and give a relative measure of the RNA purity.

RNA concentration. The concentration of RNA can be determined by measuring the optical absorbance at 260 nm (A_{260}) with a spectrophotometer. Dilute the samples in TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) for accurate spectrophotometry. Samples with A_{260} less than 0.10 are subject to increasing experimental variance. A reading of 1 absorbance unit at A_{260} in a 1-cm cuvette is equivalent to 40 µg RNA per milliliter.

RNA purity. The ratio of A_{260}/A_{280} and A_{260}/A_{230} provide a measure of nucleic acid purity. Pure RNA generally has an A_{260}/A_{280} about 2.0 (DNA is closer to 1.8), however, ratios between 1.8 and 2.2 are accepted as normal variation. A_{260}/A_{280} below 1.8 are typically indicative of protein contamination. Polysaccharides and other organic molecules, such as phenol and guanidine, absorb strongly in the 230-nm range. Pure nucleic acids have an A_{260}/A_{230} of 2.0; lower ratios indicate contamination with polysaccharides or other organic compounds. RNA preparations with A_{260}/A_{280} or A_{260}/A_{230} below 1.8 should be used with caution.

For a detailed protocol regarding spectrophotometry of RNA, see "Molecular Cloning. A Laboratory Manual", Volume 3, Section A8.20 (Sambrook & Russel, 2001).

Agilent 2100 Bioanalyzer

We recommend using the Agilent 2100 Bioanalyzer with the appropriate RNA analysis kit for assessing RNA yield and quality. This method permits analysis of RNA size, distribution, and concentration in a manner similar to gel electrophoretic methods.

For plant cell or tissue samples, high quality total RNA should exhibit clear, sharp ribosomal RNA bands. The number of ribosomal bands can vary between species and tissue types. For example, photosynthetic tissue, such as leaf tissue, contains several smaller ribosomal RNAs from the chloroplast in addition to the cytosolic 25S and 18S rRNAs bands. Non-photosynthetic tissue, such as seed or root tissue, do not contain these extra plastid rRNA bands. Quality plant total RNA should have sharp, distinct rRNA peaks (see **Figure 2**). High baseline and indistinct peaks are indicative of RNA degradation. Messenger RNA from plants typically accounts for only 0.1% of the total RNA and is usually not seen in an electropherogram. The apparent integrity of a total RNA sample can vary with sample collection and processing, as well as inappropriate storage and sample handling.

For More Information

More information about the RNA analysis assays and the Agilent 2100 Bioanalyzer can be found at:
http://www.agilent.com/chem/chip

References

1 "Molecular Cloning. A Laboratory Manual", Volume 3, Section A8.20 (Sambrook & Russel, 2001).

2 "Current Protocols in Molecular Biology," Section 4.9 (Ausubel et al., eds., 1994).

Additional Information

For additional information regarding Plant RNA Isolation, see "High-Purity RNA Isolated from a Wide Range of Plant Species and Tissue Types Using the Agilent Plant RNA Isolation Mini Kit", publication number 5989-2271EN.

5 Troubleshooting

Table 5 below lists several common symptoms that can result from processing techniques, and suggests appropriate resolutions.

Table 5 Common problems and suggested solutions

Symptom	Suggestion
Prefiltration column clogged	Reduce viscosity with one of the following procedures: • Dilute homogenate with additional Extraction Solution. • Homogenize the lysate more thoroughly. • Use less of the starting material. • Centrifuge the lysate before prefiltering.
RNA yield is low	• Ensure that the disruption and homogenization is sufficient. Grind the tissue samples thoroughly. • Purify RNA as soon as the lysate is prepared. If using lysates stored at –70 °C, thaw at 37 °C for **15–20 minutes** before use. • Check the sample integrity. Ensure that the tissue is frozen in liquid nitrogen **immediately** after separation from the organism. • Ensure that the columns (both mini prefiltration and mini isolation) are not overloaded with the sample. • Increase the amount of starting material, since the expected yields of RNA differ widely between species and tissue types.
RNA is degraded	• Closely follow the RNase free technique for cleaning equipment (see "RNase-Free Technique" on page 12). • Ensure that the tissue is not thawed before immersion in Extraction Solution. • Check the sample integrity. Ensure that the tissue is homogenized **immediately** after harvest or is **immediately** frozen in liquid nitrogen. • Purify RNA as soon as the lysate is prepared.
A_{260}/A_{280} Ratio is low	The A_{260}/A_{280} ratio is influenced by the pH of the sample. Reliable measurements can be made in buffered solutions such as TE pH 8.0, rather than water.

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